Biochem. J. (1979) **178**, 313–322
Printed in Great Britain

Turnover of Prolyl Hydroxylase Tetramers and the Monomer-Size Protein in Chick-Embryo Cartilaginous Bone and Lung *in vivo*

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(*Received* 21 *July* 1978)

The turnover of prolyl hydroxylase and an immunoreactive protein that corresponds in size to the smaller subunit of the enzyme was studied *in vivo* after injection of [^{3}H]leucine into 11-day chick embryos. The specific radioactivity and total radioactivity of the monomer-size protein were much higher than those of the enzyme tetramers in the cartilaginous bone at 3h and 12h after the radioisotope injection, indicating that the monomer-size protein represents precursors rather than degradation products of the enzyme tetramers. Between 24 and 144h after the injection the specific radioactivity and total radioactivity of the two forms of the enzyme protein showed essentially identical decay rates, the observed specific radioactivity of the monomer-size protein being about 120–130% and total radioactivity about 80% of that of the enzyme tetramers. The true half-life, when corrected for dilution caused by tissue growth and re-utilization of the [^{3}H]leucine, was 37.9h for the monomer-size protein and 39.0h for the tetramers. The results obtained in the lung were less reliable owing to high blank radioactivity values in the immunoprecipitation, but even so some definite differences were found between this tissue and the cartilaginous bone. The specific radioactivity of both forms of the enzyme protein at 24h was only about 20–25% of that in the cartilaginous bone. The total radioactivity of the monomer-size protein in the lung remained about 5 times that of the enzyme tetramers, whereas it was only about 0.8 times that of the tetramers in the cartilaginous bone. As in the cartilaginous bone, the decay rates of both forms of the enzyme protein were essentially identical in the lung, with a true half-life of about 46h. The results suggest that the rate of prolyl hydroxylase synthesis is slower in the lung than in the cartilaginous bone, whereas the degradation rates are fairly similar in these two tissues. The data further suggest that, in the lung at least, a large part of the monomer-size protein became degraded without being converted into enzyme tetramers.

Prolyl hydroxylase catalyses the synthesis of 4-hydroxyproline in collagen by the hydroxylation of prolyl residues in peptide linkages. The active enzyme is a tetramer with a mol.wt. of about 240000 and consists of two different types of monomer with mol.wts. of about 60000 and 64000 (for reviews see Cardinale & Udenfriend, 1974; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976).

Immunoreactive prolyl hydroxylase protein is present in cultured cells (McGee & Udenfriend, 1972; Stassen *et al.*, 1973; Kao *et al.*, 1975) and in intact tissues (Stassen *et al.*, 1974; Tuderman, 1976; Fuller *et al.*, 1976; Tuderman & Kivirikko, 1977) in two forms, the active enzyme tetramers and an inactive form the size of which corresponds to that of the enzyme monomers obtained on gel filtration. Preliminary characterization of the monomer-size protein from chick-embryo tendon cells by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated the presence of one major polypeptide chain having the same mobility as the smaller subunit of the enzyme and one minor one, which represented about 5–10% of the immunoreactive monomer-size protein and had the same mobility as the larger subunit (Tuderman *et al.*, 1977). Initially it was considered possible that most of the larger subunit may have been lost during the steps after homogenization of the cells (Tuderman *et al.*, 1977). However, the immunoreactive monomer-size protein in newborn-rat skin was likewise shown to consist essentially of only one polypeptide chain, which had the same size as the smaller subunit of the enzyme, and many control experiments seemed to rule out the possibility of the larger subunit being present in the newborn-rat skin in significant amounts but being subsequently lost (Chen-Kiang *et al.*, 1977). The monomer-size protein was purified from newborn-rat skin to near homogeneity and shown to be identical with, or very

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similar to, the smaller subunit of prolyl hydroxylase by a variety of techniques, including amino acid analysis and reverse-phase chromatography of peptides obtained by tryptic digestion (Chen-Kiang *et al.*, 1977).

The metabolic relationship between prolyl hydroxylase tetramers and the monomer-size protein in the cells is not known. In most cells and tissues the concentration of the monomer-size protein is much higher than that of the enzyme tetramers, and the ratio between these two forms has been found to vary markedly between different tissues and in the same tissue in different physiological and pathological states (Stassen *et al.*, 1974; Tuderman, 1976; Risteli *et al.*, 1976, 1978; Tuderman & Kivirikko, 1977). The tetramers can be dissociated into the monomers in isolated cells with dithiothreitol, and are re-formed during further incubation without dithiothreitol in the absence of protein synthesis (Stassen *et al.*, 1974; Tuderman *et al.*, 1977). These results indicate that at least under certain conditions the monomers can be associated into the active tetramers after their release from ribosomes. However, the data do not indicate whether the monomer-size protein in the cells normally represents a precursor or degradation product of the enzyme tetramer, or both. Experiments involving the labelling of prolyl hydroxylase protein in isolated cells (Tuderman *et al.*, 1977) or in neonatal-rabbit skin *in vivo* (Chichester *et al.*, 1976) suggest that the monomer-size protein represents, at least in part, precursors of the enzyme tetramers. The results obtained in these studies (Chichester *et al.*, 1976; Tuderman *et al.*, 1977) further suggest that the turnover rates of both forms of the enzyme protein are relatively slow; in neonatal-rabbit skin the half-life of the tetramers was about 78h and that of the monomer-size protein about 44h (Chichester *et al.*, 1976). The values were not corrected for the influence of re-utilization of the labelled amino acid or for dilution of the enzyme protein, owing to tissue growth, however (Chichester *et al.*, 1976). Furthermore, direct comparison of the specific activities of the tetramers and the monomer-size protein was not possible, as different units were used for these two forms (Chichester *et al.*, 1976). In the present work an attempt was made to study further the relationship between the two forms of prolyl hydroxylase protein by measuring their turnover rates in chick-embryo cartilaginous bone and lung *in vivo*.

Experimental

Chick embryos and preparation of cartilaginous bone and lung extracts

Fertilized eggs of White Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and incubated in a moist atmosphere at 37°C. On day 10 they were tested for fertility by inspection with a lamp, and 200μCi of L-[4,5-^{3}H]leucine (40000–60000Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.2ml of 0.1M-NaCl was injected on to the chorioallantoic membrane through a small hole in the shell. The hole was covered with adhesive tape and the incubation continued. The embryos were killed in groups of two at 3, 12, 24, 48, 72 and 96h after the injection in Expt. I and at 24 and 144h in Expt. II. The embryos were dried gently with filter paper and rapidly weighed, and then the humeri, ulnae, radii, femurs and tibiae were carefully dissected, weighed and stored at −70°C. The pooled bones of one embryo constituted one sample of cartilaginous bone. The lung tissue was also carefully prepared and stored at −70°C.

Two bone or lung samples were thawed at a time and homogenized with an Ultra-Turrax homogenizer for 3×5s in a cold (0°C) solution consisting of 0.1M-NaCl, 0.1M-glycine, 0.1% (w/v) Triton X-100 and 20mM-Tris/HCl buffer, adjusted to pH7.5 at 4°C (4ml of solution/g of tissue). The homogenates were incubated at 4°C for 1h with occasional shaking and then centrifuged at 15000*g* for 30min at 4°C. The supernatant constituted the cartilaginous bone or lung extract.

Separation of prolyl hydroxylase tetramers and the monomer-size protein

The two tissue extracts prepared on the same day were applied to two gel-filtration columns (1.5cm×90cm) of 8% agarose (Bio-Gel A-1.5m; Bio-Rad, Bromley, Kent, U.K.), which were equilibrated and eluted with a solution containing 0.1M-NaCl, 0.1M-glycine and 0.01M-Tris/HCl buffer, adjusted to pH7.5 at 4°C. Fractions (3ml) were collected and assayed for immunoreactive prolyl hydroxylase content. On the next day two other tissue extracts were prepared and applied to the two gel-filtration columns as described above. Thus four tissue extracts were always fractionated by gel filtration in 2 days.

Two or three of the gel-filtration fractions with the highest amounts of prolyl hydroxylase tetramers or the monomer-size protein were pooled, thus forming four tetramer and four monomer-size protein pools. These pools were concentrated by ultrafiltration with PM-30 membranes in Amicon ultrafiltration cells (Amicon Corp., Lexington, MA, U.S.A.) to contain about 2–6μg of prolyl hydroxylase tetramers or of the monomer-size protein of the cartilaginous bone/ml or about 0.7–2.0μg of those of the lung/ml. The concentrated pools were centrifuged at 15000*g* for 10min, and a sample of each supernatant was taken for an assay of immunoreactive prolyl hydroxylase protein. These values indicated the actual amounts of the enzyme protein taken for immunoprecipitation,

and thus varying losses during previous steps should not affect the specific activities. Other samples were taken for immunoprecipitation, which was carried out immediately after the centrifugation.

Immunoprecipitation

Rabbit immunoglobulins were precipitated with 40%-satd. $(NH_4)_2SO_4$ from 5ml of antiserum against pure chick prolyl hydroxylase (Kuutti *et al.*, 1975) and coupled to 5ml of 4% agarose (Sepharose 4B; Pharmacia Fine Chemicals, Uppsala, Sweden) by using the CNBr activation technique (Cuatrecasas & Anfinsen, 1971). This preparation was termed antibody–agarose. Another preparation, termed non-antibody–agarose, was prepared in a similar manner from 5ml of rabbit serum not containing antibody against prolyl hydroxylase.

Immunoprecipitation was carried out in a final volume of 1.0ml containing 0.3ml of the concentrated prolyl hydroxylase tetramer or the monomer-size protein pool, 0.1ml of antibody–agarose or non-antibody–agarose and 0.6ml of a solution containing 0.15M-NaCl, 2.5mg of bovine serum albumin/ml and 0.01M-Tris/HCl buffer, adjusted to pH7.8 at 25°C (Tuderman *et al.*, 1975). The eight samples (containing antibody–agarose) and eight control samples (containing non-antibody–agarose) were incubated with shaking at room temperature (21°C) for 1h, and the immunoprecipitates separated by centrifugation at about 300*g* for 2min, washed with four 2ml portions of the above solution, suspended in 1.5ml of water and counted for radioactivity with 5ml of Instagel (Packard, Caversham, Berks., U.K.).

Measurement of the concentration of immunoreactive prolyl hydroxylase protein

The amount of immunologically cross-reacting prolyl hydroxylase protein was measured by using a specific radioimmunoassay based on the inhibition by non-labelled enzyme of complexing of ^{3}H-labelled pure chick-embryo prolyl hydroxylase by antibody and subsequent precipitation of the enzyme–antibody complex by a cellulose-bound second antibody (Tuderman *et al.*, 1975). Pure chick-embryo prolyl hydroxylase was used as the standard enzyme, and values were expressed as μg of pure prolyl hydroxylase (Tuderman *et al.*, 1975). The ratios of bound/total radioactivity were corrected for the ^{3}H label present in the enzyme to be analysed by using values obtained in the immunoprecipitation. In most instances the effect of this correction was negligible, and in no instance did its magnitude exceed 10% of the original value.

It has previously been demonstrated that monomers prepared from prolyl hydroxylase tetramers by dissociation with dithiothreitol are equivalent to the tetramers in this assay (Tuderman *et al.*, 1975). On this basis it was assumed that the monomer-size protein also behaves in an identical fashion, but it was not possible to carry out experiments to test the validity of this assumption.

Other assays

In order to characterize the monomer-size prolyl hydroxylase protein, immunoprecipitation was carried out as above, and the washed immunoprecipitate incubated with 200μl of 0.125M-Tris/HCl, pH6.8 at 22°C, containing 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol and 0.001% Bromophenol Blue at 37°C for 2h. After centrifugation at about 1000*g* for 10min, the supernatant was dialysed against the above buffer overnight, reduced by adding 5% (w/v) of β-mercaptoethanol, boiled for 3min and immediately applied to slab gels. Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was carried out by the procedure of King & Laemmli (1971), by using 10% (w/v) polyacrylamide separating gel. The radioactive bands in the gels were then located by fluorography (Bonner & Laskey, 1974).

The decay in radioactivity in the free leucine pool of the chick embryo was studied by injecting 5μCi of [4,5-^{3}H]leucine on to the chorioallantoic membrane in 0.2ml of 0.1M-NaCl as described above. At the time points indicated in Fig. 4 (see the Results section), the embryos were killed, weighed and homogenized in cold (0°C) 0.15M-NaCl with an Ultra-Turrax homogenizer (1ml of solution/g of the embryo). The homogenates were centrifuged at 15000*g* for 30min, and 1ml of 20% (w/v) trichloroacetic acid was added to 1ml samples of the supernatants. The samples were left for 1h, and then centrifuged at 15000*g* for 10min. The supernatants were extracted with 3 × 2vol. of diethyl ether to remove the trichloroacetic acid, and 1ml portions of the aqueous phase were then freeze-dried (Lebherz, 1975). The residues were taken up in 1.5ml of water and counted for radioactivity with 5ml of Instagel (Packard). Other 2ml samples of the chick-embryo extract received 50mg of solid sulphosalicylic acid. The supernatants were separated by centrifugation at 15000*g* for 10min, and 1ml samples were then used to analyse the leucine content in a Jeol JLC-5AH automatic amino acid analyser.

All radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 35% and a background of 10c.p.m. for ^{3}H radioactivity.

The regression lines shown in Figs. 1, 3, 5, 6 and 7 were fitted by means of a least-square regression analysis to an exponential curve, and those in Fig. 2 by linear-regression analysis.

Results

Changes in the specific radioactivity of prolyl hydroxylase tetramers and the monomer-size protein in the cartilaginous bone

Preliminary experiments indicated that both forms of prolyl hydroxylase protein slowly form precipitates at 4°C, about 10–15% of the soluble immunoreactive protein precipitating out in 1 week. It was for this reason that the frozen samples were analysed in series of four as described in the Experimental section. Immunoprecipitation was carried out immediately after centrifugation of the concentrated final pools, as the amount of non-specific precipitation also increases with time. Considerable losses of the enzyme protein (up to 30%) occurred during the steps of the purification procedure, especially during the concentration and subsequent centrifugation of the pools obtained by gel filtration. This should not affect the specific activities, however, provided that the losses in radioactive and non-radioactive prolyl hydroxylase protein were similar, as the amount of enzyme protein was determined again in samples of the final pools. Immunoprecipitation recovery was tested by mixing labelled and non-labelled pure prolyl hydroxylase in various combinations. About 70% of the labelled enzyme was found in the immunoprecipitates when 0.5–2.0 μg of enzyme protein was precipitated and washed as described in the Experimental section. Additional experiments indicated that less than 10% of the tetramers or the monomer-size protein remained in the supernatant when the final pools from the cartilaginous bone or lung enzyme were immunoprecipitated.

The specificity of immunoprecipitation was controlled by using precipitation with non-antibody–agarose. About 0.5% of the total radioactivity in the final pools from cartilaginous bone was found in these precipitates. This non-specific precipitation was about 10–15% of that found with the antibody–agarose, there being no differences between the tetramer and the monomer-size protein pools, nor between the early and late time points after injection of the labelled leucine. The non-specific-precipitation values were subtracted from the final values.

The specificity of the immunoprecipitation was further controlled by examining the immunoprecipitates of the monomer-size protein pool by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis. Only one major radioactive band and two minor bands were found, the mobility of the major band corresponding to that of the smaller subunit of the enzyme (results not shown). This band represented about 90–95% of total radioactivity found in the gel. One minor band was at the dye front and one very weak band in the position of the larger subunit of the enzyme.

Two series of experiments were carried out, the first containing twelve and the second four embryos. As no significant differences were found between the two experiments, their results are shown in the same Figures, but values obtained in Expt. I are denoted by circles, and those in Expt. II by squares.

The specific radioactivity of the monomer-size protein (Fig. 1, closed symbols) was about 2.7 times that of the enzyme tetramers (Fig. 1, open symbols) at 3h after [^{3}H]leucine injection. During the subsequent 21h, a small decrease occurred in the specific radioactivity of the monomer-size protein, and a small increase in that of the tetramers, so that at 24h the specific radioactivity of the monomer-size protein was only about 1.3 times that of the tetramers. After this time point the rates of decay of the specific radioactivities of both forms of the enzyme protein were similar. The specific radioactivity of the monomer-size protein thus remained about 20–30% higher than that of the tetramers (Fig. 1). The apparent half-lives were calculated by using the equation (see Schimke & Doyle, 1970; Poole, 1971; Rechcigl, 1971):

$$t_{\frac{1}{2}} = \frac{\ln 2}{k'}$$

in which $t_{\frac{1}{2}}$ is the apparent half-life to be calculated, and k' the decay rate obtained from the regression line. A value of about 40h was obtained for both forms of the enzyme protein (tetramers 41.7h, monomer-size protein 40.2h).

Changes in the total radioactivity of prolyl hydroxylase tetramers and the monomer-size protein in the cartilaginous bone

To obtain further information on the metabolic relationship between the two forms of immunoreactive prolyl hydroxylase protein, the radioactivity values were expressed as total activities per whole cartilaginous bone sample. Previous (Tuderman, 1976) and additional values were used to prepare curves showing the amounts of prolyl hydroxylase tetramers and the monomer-size protein in ng/mg of cartilaginous bone for embryos of various weights. These values were then multiplied by the weights of the cartilaginous bone in each sample of the present experiments. The values obtained are shown in Fig. 2. Most of the increases in the amounts of both forms of the enzyme protein were due to an increase of about 4.3-fold in the bone weight between 3 and 144h of the experiment. The concentration of enzyme tetramers per mg of bone at 144h was about 1.4-fold and that of the monomer-size protein about 1.2-fold, the tetramers forming about 58–64% of the total enzyme protein at various time points.

The total radioactivities of the two forms of the enzyme protein were calculated by multiplying their amounts (Fig. 2) by their specific radioactivities (Fig.

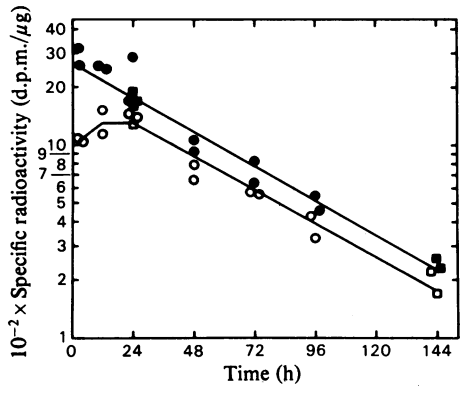

Fig. 1. *Specific radioactivities of prolyl hydroxylase tetramers and the monomer-size protein in cartilaginous bone*
[^{3}H]Leucine (200μCi) was injected on to the chorio-allantoic membrane of 11-day chick embryos. The embryos were killed at 3–144h after the injection, and prolyl hydroxylase tetramers and the monomer-size protein of cartilaginous bone extracts were purified by gel-filtration and immunoprecipitation as described in the Experimental section. The specific radioactivities of the immunoprecipitates were corrected for non-specific precipitation as described in the text. The regression lines were calculated for both forms of the enzyme protein between 24 and 144h, and the apparent half-lives were then determined from these lines (tetramers, Pearson correlation coefficient $r = -0.979$, apparent half-life 41.7h; monomer-size protein, $r = -0.971$, apparent half-life 40.2h). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

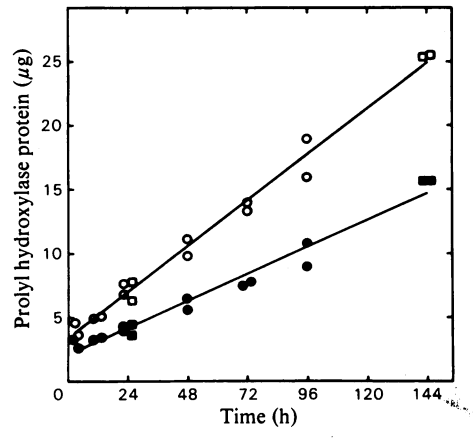

Fig. 2. *Amounts of prolyl hydroxylase tetramers and the monomer-size protein in the cartilaginous-bone samples*
The values were calculated by preparing curves showing the amounts of prolyl hydroxylase tetramers and the monomer-size protein in ng/mg of cartilaginous bone for embryos of various weights. These values were then multiplied by the weights of the cartilaginous bone in each sample of the present experiments. The regression lines were calculated for both forms of the enzyme protein between 3 and 144h (tetramers, $r = 0.994$; monomer-size protein, $r = 0.986$). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

1), and these values are shown in Fig. 3. A definite increase was observed in the total radioactivity of the enzyme tetramers between 3 and 24h, whereas that of the monomer-size protein showed little change. After 24h the total radioactivities showed similar decay rates, with apparent half-lives of about 115h (tetramers 118h, monomer-size protein 114h). The total radioactivity of the monomer-size protein was higher initially than that of the tetramers, but remained subsequently at about 80% of the total activity of the tetramers (Fig. 3).

Decay of radioactivity in the free-leucine pool of the chick embryo

Re-utilization of the labelled leucine can have marked influence on the decay of radioactivity in the protein; an apparent half-life of about 3.5 days has been reported for catalase in rat liver without correction for re-utilization of the labelled amino acid, for instance, whereas the true value obtained with such a correction is only 1.5 days (see Poole, 1971). The decay in the radioactivity in the free-leucine pool was studied in the present case by injecting 14 embryos

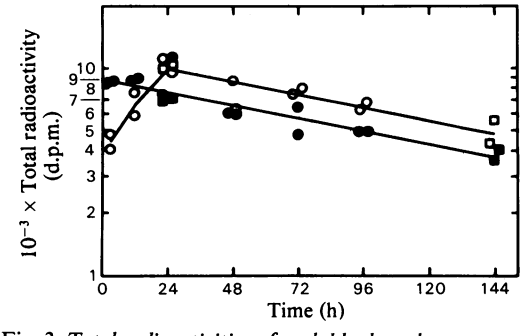

Fig. 3. *Total radioactivities of prolyl hydroxylase tetramers and the monomer-size protein in cartilaginous bone*
The values were calculated by multiplying the amounts (Fig. 2) by the specific activities (Fig. 1). The regression lines were calculated for both forms of the enzyme protein between 24 and 144h (tetramers, $r = -0.916$, apparent half-life 118h; monomer-size protein, $r = -0.879$, apparent half-life 114h). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

with 5μCi of [^{3}H]leucine in 0.2ml of 0.1M-NaCl, the injection being identical with that used in the above experiments except that the amount of radioactivity was decreased to one-fourtieth. This decrease should

cause no difference in the decay rate, as the quantity of [^{3}H]leucine injected was in both cases negligible compared with the size of the free-leucine pool of the embryo. The trichloroacetic acid-soluble radioactivity was measured as described in the Experimental section, the procedure involving freeze-drying of the samples (Lebherz, 1975). This step removes the [^{3}H]water formed from the [^{3}H]leucine. The values for the non-volatile trichloroacetic acid-soluble radioactivity were expressed in d.p.m./μmol of free leucine. It should be noted that this value is not identical with the specific radioactivity of the free leucine, but the rate of decay in this value can be expected to correlate with that of the radioactivity of the free amino acid pool (Lebherz, 1975).

There was a rapid decay in the radioactivity/μmol of free leucine during the first hours after the injection (Fig. 4), followed by a slow decay during the subsequent days. The solid line in Fig. 4 represents the equation:

$$F(t) = \frac{1.5}{3+t} + \frac{2.8\times 10^6}{(0.6+t)^2}$$

where $F(t)$ is the radioactivity expressed as d.p.m./μmol of free leucine and t the time (h) after injection of the labelled amino acid. As seen in Fig. 4, this equation fits reasonably well with the experimental results.

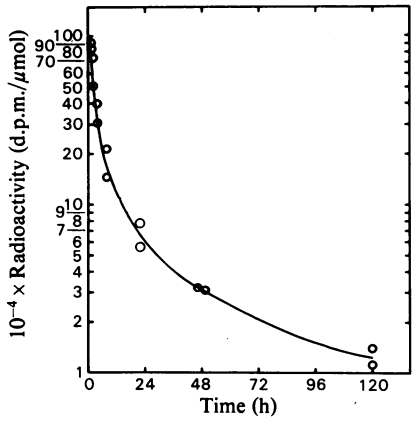

Fig. 4. *Decay of radioactivity in the free leucine pool of the chick embryo*

[^{3}H]Leucine, 5μCi, was injected on to the chorioallantoic membrane of 11-day chick embryos, and the non-volatile trichloroacetic acid-soluble radioactivity and the leucine content of the chick-embryo extract were determined, as described in the Experimental section. The values are given as d.p.m./μmol of free leucine. The solid line represents the equation given in the text.

Correction of apparent turnover rates for re-utilization of leucine

The specific radioactivities shown in Fig. 1 were corrected for the re-utilization of [^{3}H]leucine by using the equation (Poole, 1971):

$$\frac{dP(t)}{dt} = k\cdot a\,[F(t)-P(t)]$$

in which $P(t) = P(0)\cdot e^{-k't}$, $P(t)$ is the radioactivity of the tetramers or the monomer-size protein in d.p.m./μg as a function of time, t the time (h) after injection of the labelled amino acid, $F(t)$ the radioactivity in d.p.m./μmol of free leucine (see above); k the decay rate to be calculated, k' the decay rate observed, and a is a factor that corrects the value of the function $F(t)$ to that of the observed specific radioactivity of the tetramers or the monomer-size protein. The value for a was determined experimentally by studying the change in the mean specific radioactivity in Fig. 1 between 24 and 120h at 0.1h intervals. The mean value for a obtained in the cartilaginous bone at various time points was 0.01086 for the tetramers and 0.01461 for the monomer-size protein. In both cases the maximum deviation from the mean value was less than $\pm 2\%$.

By solving this equation, values were obtained for k amounting to 0.0283 for the tetramers and 0.0294 for the monomer-size protein in the cartilaginous bone. These were then used to calculate the corrected specific radioactivities at each time point between 24 and 144h, and the corrected specific radioactivities were further converted into corrected total radioactivities by multiplying them by the amounts of tetramers or the monomer-size protein, as described above. The corrected values for the total radioactivities were used to determine the true half-lives of both forms of the immunoreactive enzyme protein between 24 and 144h by fitting a line through the corrected experimental points (Fig. 5). The true half-life for the enzyme tetramers was 39.0h ($r = -0.990$) and that for the monomer-size protein 37.9h ($r = -0.984$).

Attempts to measure turnover of prolyl hydroxylase in lung

Attempts were also made to study the turnover of prolyl hydroxylase in lung and liver, these two tissues differing from the cartilaginous bone in that most of the enzyme protein is present in the monomer-size form (Tuderman, 1976). The results indicated, however, that it was not possible to study the specific radioactivity of the enzyme protein in the liver with the techniques used here, as some samples showed little difference between the amounts of radioactivity precipitated with the antibody–agarose and non-antibody–agarose.

The values obtained with the non-antibody–

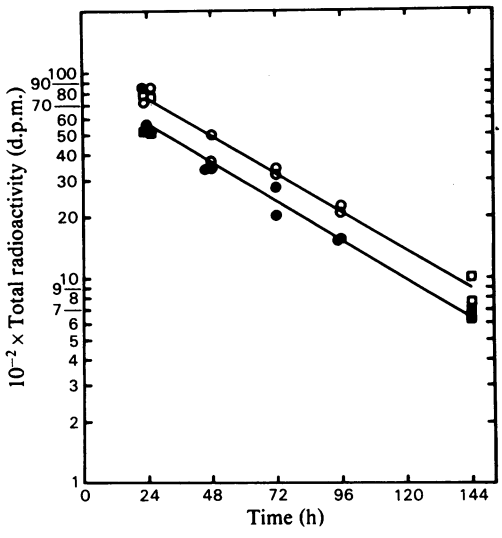

Fig. 5. *Corrected total radioactivities of prolyl hydroxylase tetramers and the monomer-size protein in cartilaginous bone*

The specific radioactivities at 24–144h (Fig. 1) were corrected for the re-utilization of [^{3}H]leucine by using the equation given in the text. The corrected specific radioactivities were further converted into corrected total radioactivities by multiplying them by the amounts of tetramers or the monomer-size protein (Fig. 2), and the regression lines were calculated for these corrected values (tetramers, $r = -0.990$, half-life 39.0h; monomer-size protein, $r = -0.984$, half-life 37.9h). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

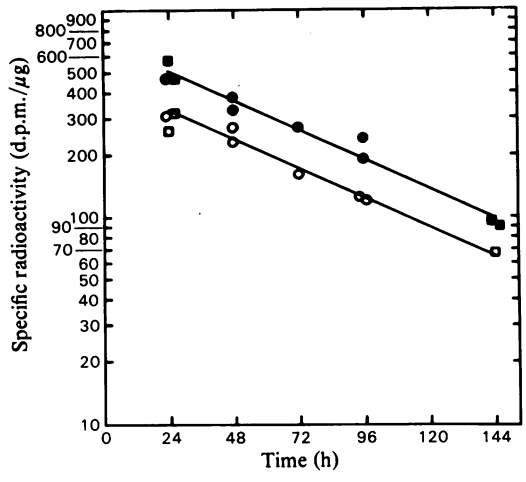

Fig. 6. *Specific radioactivities of prolyl hydroxylase tetramers and the monomer-size protein in the lung*

The lung tissue was prepared from the same embryos as the cartilaginous bone. For further details see Fig. 1 and the text. Values were not determined before 24h owing to the high values obtained with the non-antibody–agarose (see the text). Two samples were lost owing to difficulties in preparing the lungs and one sample of tetramers at 144h was lost in the gel filtration. The regression line was calculated for the tetramers between 48 and 144h ($r = -0.990$, apparent half-life 50.6h) and for the monomer-size protein between 24 and 144h ($r = -0.986$, apparent half-life 50.6 h). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

agarose were also quite high in the lung, especially during the first 12h after injection of the radioisotope, but after the first 24h the value obtained with the non-antibody–agarose became established at a rather constant fraction of the total radioactivity, about 3% for the enzyme-tetramer pools and about 1% for the monomer-size-protein pools. This non-specific precipitation was about 50% of that found with the antibody–agarose in the enzyme-tetramer pools and about 25% in the monomer-size-protein pools. The specific radioactivities shown for the two forms of the immunoreactive enzyme protein in the lung (Fig. 6) were calculated by subtracting the radioactivities found with the non-antibody–agarose in each pool from those obtained with the antibody–agarose. The values were then converted into total radioactivities (Fig. 7) by using a similar procedure to that described above for the cartilaginous bone. The enzyme tetramers formed about 22% of the total immunoreactive enzyme protein at all time points.

The values for the radioactivity of the two forms of the immunoreactive enzyme protein in the lung, especially for the enzyme tetramers, may be subject to considerable inaccuracies, since the magnitude of the non-specific precipitation was quite high. Some definite differences can nevertheless be seen between the values in the lung and the cartilaginous bone. The specific radioactivity of both forms of the enzyme protein at 24h in the lung was only 20–25% of that in the cartilaginous bone (compare Figs. 1 and 6). The total radioactivity of the monomer-size protein in the lung was about 5 times that of the tetramers between 24h and 144h, whereas in the cartilaginous bone it was about 0.8 times that of the tetramers (compare Figs. 3 and 7). The true half-lives of the total radioactivities of the two forms of the immunoreactive enzyme protein in the lung, when corrected for re-utilization of leucine as described above, were 46.0h ($r = -0.989$) for the enzyme tetramers between 48h and 144h, and 45.9h ($r = -0.970$) for the monomer-size protein between 24h and 144h (results not shown).

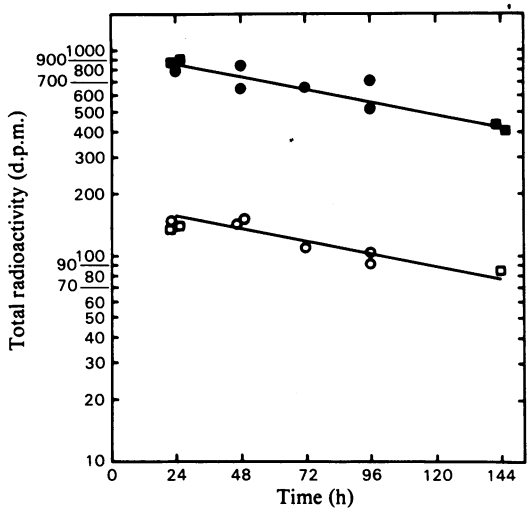

Fig. 7. *Total radioactivities of prolyl hydroxylase tetramers and the monomer-size protein in the lung*
The specific radioactivities (Fig. 6) were converted into total radioactivities by using a similar procedure to that described for the cartilaginous bone (Figs. 2 and 3). The regression line was calculated for the tetramers between 48 and 144h ($r = -0.926$, apparent half-life 118h) and for the monomer-size protein between 24 and 144h ($r = -0.927$, apparent half-life 123h). ○, Tetramers in Expt. I; □, in Expt. II. ●, Monomer-size protein in Expt. I; ■, in Expt. II.

Discussion

The main aim of the present study was to examine the metabolic relationship between prolyl hydroxylase tetramers and the immunoreactive monomer-size protein *in vivo*. At 3h and 12h after injection of the radioisotope the specific radioactivity and total radioactivity of the monomer-size protein in cartilaginous bone were much higher than those of the enzyme tetramers, indicating that the monomer-size protein represents precursors rather than degradation products of the enzyme tetramers. The results at these early time points are similar to those observed in pulse–chase experiments with isolated chick-embryo tendon cells (Tuderman *et al.*, 1977). After the first 24h, the specific radioactivities and total radioactivities of the monomer-size protein and enzyme tetramers in the cartilaginous bone showed essentially identical decay rates. In the lung, the linear decay of the specific and total radioactivities of the enzyme tetramers was observed only at 48h, but again the decay rates of the two forms of the enzyme protein were identical after this time point. The specific radioactivity of the monomer-size protein remained higher than that of the enzyme tetramers in both tissues during the whole observation period. This may be due in part to re-utilization of the labelled leucine, but it may also indicate that at least some of the label in the monomer-size protein pool is not transferred to the enzyme tetramer pool.

Comparison of the total radioactivity of the two forms of the immunoreactive enzyme protein between cartilaginous bone and lung gave further support for the latter suggestion. In cartilaginous bone the total radioactivity of the monomer-size protein was lower than that of the tetramers after about 20h, whereas in the lung the total radioactivity of the monomer-size protein remained markedly higher than that of the tetramers during the whole experiment. It thus seems that, in lung at least, a large part of the labelled monomer-size protein becomes degraded without being converted into enzyme tetramers.

The data also made it possible to calculate the true half-lives of the two forms of the immunoreactive enzyme protein. Since the amounts of these proteins increased markedly during the experiment, the decay rates were calculated for the total radioactivities. Correction for re-utilization of the labelled leucine had a substantial effect on the observed half-lives, this result being similar to that reported for other enzymes (Poole, 1971). The true half-life found for both forms of the enzyme protein was about 38–39h in cartilaginous bone and about 45–46h in lung. As noted in the Results section, the values obtained in the lung may be subject to considerable inaccuracies, and it thus seems that the minor difference in true half-life between the cartilaginous bone and lung cannot be regarded as significant. By contrast, the marked difference between the cartilaginous bone and lung in the specific radioactivities of both forms of the enzyme protein is clearly a true difference. As the specific radioactivity of the free leucine pool was not measured directly in the lung tissue, it is not known whether there are significant differences in the isotope dilution between the cartilaginous bone and lung. Such differences could account for the differences in the specific radioactivities of the enzyme protein between these two tissues. However, it seems more likely that these differences were due to a slower rate of prolyl hydroxylase synthesis in the lung than in the cartilaginous bone. Accordingly, the lower amount of enzyme protein in the lung than in the cartilaginous bone is probably due primarily to a slower rate of prolyl hydroxylase synthesis rather than a faster rate of enzyme degradation. This suggestion agrees with reports on the regulation of the amounts of several other enzymes in vertebrate tissues (Rechcigl, 1971).

The present half-lives differ from the apparent half-lives determined in the skin of neonatal rabbits in that the decay rates of the two forms of the enzyme protein were found to be dissimilar in rabbit skin (Chichester *et al.*, 1976). The apparent half-life of the enzyme tetramers was about 78h and that of the monomer-size protein about 44h, without correction

for the dilution caused by tissue growth or for the re-utilization of the labelled leucine (Chichester *et al.*, 1976). The reason for this difference is not known, but there was a considerable scatter in the observed values in the rabbits, and it does not seem impossible that the half-lives of both forms of the enzyme protein might also have been identical in the rabbit skin. The true half-lives reported here indicate that the turnover rate of prolyl hydroxylase is relatively slow, a finding that agrees with results obtained in neonatal-rabbit skin (Chichester *et al.*, 1976) and freshly isolated chick-embryo tendon cells (Tuderman *et al.*, 1977).

The biological function of the monomer-size prolyl hydroxylase protein remains to be elucidated. The present and previous (Tuderman *et al.*, 1977; Chen-Kiang *et al.*, 1977) data suggest that this protein probably consists mainly or entirely of the smaller subunit of prolyl hydroxylase, and thus the enzyme activity cannot be controlled simply by a regulation of the association of the subunits from this pool. One possibility is that it is mainly the synthesis of the larger subunit that is regulated, this subunit then combining with the smaller subunit present in the monomer-size protein pool. Previous demonstrations that the monomers can be associated into active tetramers after their release from the ribosomes (Stassen *et al.*, 1974; Tuderman *et al.*, 1977) are consistent with this possibility. This would also explain the marked variation in the ratio of the amount of enzyme tetramers to total enzyme protein between different tissues and in the same tissue in different physiological and pathological states (Stassen *et al.*, 1974; Tuderman, 1976; Risteli *et al.*, 1976, 1978; Tuderman & Kivirikko, 1977). In normal rat liver, for example, about 4% of total prolyl hydroxylase protein is present as enzyme tetramers and 96% as the monomer-size protein, but after severe hepatic injury induced with dimethylnitrosamine the amount of tetramers increased about 4-fold in 4 days, with only minor changes in the amount of the monomer-size protein and the total enzyme protein, and thus the tetramers now comprised about 16% of the total enzyme protein (Risteli *et al.*, 1978). This result might be due to increased synthesis of the larger subunit with an essentially unchanged synthesis of the smaller subunit. If the synthesis of both subunits had increased with an unaltered ratio, one would have expected to find a 4-fold increase in the monomer-size protein too.

Finally, it should be noted that the monomer-size protein is not quite identical with the smaller subunit of prolyl hydroxylase. Differences between these two proteins were found in 2 out of 37 tryptic peptides (Chen-Kiang *et al.*, 1977). These differences may be due to small variations in the extents of post-translational modifications, such as sugar additions, but the possibility is not excluded that the monomer-size protein may be a slightly different precursor form of the smaller subunit (Chen-Kiang *et al.*, 1977). The monomer-size protein is present in many cells in a very large excess over the enzyme tetramers; for instance in adult human skin the ratio of the monomer-size protein to enzyme tetramers is about 30:1 (Tuderman & Kivirikko, 1977). Thus it is possible that the monomer-size protein is not exclusively related to prolyl hydroxylase but has also some additional functions. The recently postulated function as a precursor of lysyl hydroxylase (Chen-Kiang *et al.*, 1977) does not seem possible, however, as the molecular weights of the subunits of lysyl hydroxylase are around 90000 (Turpeenniemi *et al.*, 1977).

This work was supported in part by grants from the Medical Research Council of the Academy of Finland. We gratefully acknowledge the expert technical assistance of Mrs. Ritva Savilaakso and Mrs. Lea Torvela.

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